BACILLUS STRAIN AND ANTIBIOTIC SCREENING METHOD

Background

We previously showed that the SpoIIIE protein of *Bacillus subtilis is* required for the transfer of the prespore chromosome through the asymmetric division septum that separates the prespore from its larger mother cell *(1,2)*. *spoIIIE* mutations lead to a block in sporulation, leaving the prespore with only part of a chromosome, the remainder of the chromosome being trapped in the mother cell compartment. Further analysis of *spoIIIE* mutants has revealed that the small segment of DNA that is trapped in the prespore is a fairly specific one, centred close to the origin of DNA replication, *oriC* (1,3). This implied the existence of a mechanism which imposes a specific orientation on the chromosome destined for the prespore before septation. We have recently shown that the *spoOJ* gene is required to specify this orientation (4). However, specificity is not completely lost in *spoOJ* mutants (3), so it appears that there must be at least one secondary mechanism working to determine chromosome orientation at the onset of sporulation. The assay for inhibitors of SpoOJ function described below exploits some unexpected features of this change of specificity.

The *spoOJ* gene is highly conserved in a wide range of bacteria (5) and it is related to a family of proteins required for accurate partitioning of low-copy-number plasmids found in many diverse bacteria (6,7). Our recent results strongly suggest that SpoOJ protein has a direct role in segregation of sister chromosomes during both growth and sporulation (10). However, the gene is not essential for vegetative growth, although chromosome partitioning is partially impaired (8). Most likely, this is because of the presence of a secondary partitioning system in this

organism, perhaps the same one that we have detected in the experiments mentioned above. Nevertheless, there is at least one report of a chromosomal *spoOJ*-like gene being essential (9), consistent with the vital importance of chromosome partitioning mechanisms for bacterial viability. Thus, the *spoOJ* family of proteins may be good targets for antimicrobial agents .

The Invention

The effects of *spoOJ* mutations on prespore chromosome orientation, and the ability to detect this by use of a *spoIIIE* mutant background, provides the potential for a very specific whole-cell assay for inhibitors of SpoOJ function. The presence of any given segment of chromosomal DNA in the prespore can be detected by use of a reporter gene controlled by a transcription factor, σ^F, which is activated only in the small prespore compartment (a process that is not affected by perturbations in chromosome partitioning).

Thus the invention provides in one aspect a *Bacillus* strain having a chromosome with the following modifications:

a) a mutation of a *spoIIIE* gene which blocks transfer of the prespore chromosome,

b) a mutation which prevents loss of SpoOJ function from blocking sporulation, together with

c) a first reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to increased trapping and hence to increased expression in the prespore, and/or

d) a second reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to reduced trapping and hence to reduced expression in the prespore.

In another aspect the invention provides a method of determining whether an agent inhibits SpoOJ function in *Bacillus* species, which method comprises inducing the *Bacillus* strain as described to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and/or the second reporter gene.

In another aspect the invention provides a method which comprises inducing the *Bacillus* strain as described to sporulate in the presence of an agent, observing expression of the first and/or second reporter gene and thereby determining that the agent inhibits SpoOJ function in the *Bacillus* species, and using the agent as an antibiotic to kill or inhibit the growth of bacteria.

Preferably both the first reporter gene c) and the second reporter gene d) are present in the *Bacillus* strain of the invention. Preferably each of the first reporter gene c) and the second reporter gene d) is fused to a σ^F dependent gpr promoter. Preferably each of the first reporter gene c) and the second reporter gene d) expresses a different detectable enzyme.

In the method of the invention, expression of the first and second reporter genes is preferably observed by monitoring changes in the levels of or the ratio of their products. Preferably the first and second reporter genes are expressed as enzymes whose activities are observed by any convenient means e.g. fluorimetry or spectrophotometry. Preferably the *Bacillus* strain is induced to sporulate and is contacted, just prior to asymmetric cell division, with the agent being investigated. The method can conveniently be performed as a screening test for putative antimicrobial agents.

Any *Bacillus* species may be used that is capable of

sporulating under suitable conditions and for which genetic constructions can be made. *B subtilis* is conveniently accessible and well characterised and is preferred.

Experiments with reporter genes placed at many locations in the chromosome have shown that in a *spoIIIE* mutant a fairly specific segment of DNA is trapped in the prespore compartment (1, 3). Loss of the *spoOJ* gene results in a change in the specificity of the segment of DNA that is trapped, which leads to characteristic changes in the levels of expression of σ^F-dependent reporter genes placed at different chromosomal locations. Figure 1 shows the effect of a *spoOJ* mutation on expression of a σ^F-dependent reporter gene (*gpr-lacZ*) placed at different chromosomal locations in a *spoIIIE36* background. To control for day to day variation in absolute β-galactosidase levels, each experiment was done with a control strain comprising the reporter at the same location in a *spoIIIE+* background. Each activity is expressed relative to that of the corresponding control. Thus, for example when a *spoOJ* mutation is combined with the *spoIIIE* mutation, expression of the reporter gene increases at the *sigL* location but decreases at the *amyE* location. Loss of SpoOJ function can therefore be detected by the large change that characteristically occurs in the levels of the products of the two reporters.

A preferred assay strain (e.g. 1238) contains several genetic modifications. First, a point mutation in the *spoIIIE* gene to block transfer of the prespore chromosome. The *spoIIIE36* mutation is a convenient, well characterised, example of the appropriate type (1, 2). Second, a *soj* mutation, to prevent the loss of SpoOJ function from blocking sporulation (8). Any mutation abolishing *soj* function without unduly affecting expression of the adjacent *spoOJ* gene; such as the large in-frame deletion constructed by Ireton *et al* (8) would be appropriate. Third, a reporter gene, *lacZ,* fused to the σ^F-dependent *gpr* promoter and placed at the *sigL* location, where impaired SpoOJ function leads to increased

trapping in the prespore and thus increased synthesis of the reporter gene product, β-galactosidase. Fourth, a second reporter gene, similar to the first but placed at the *amyE* location, where trapping and thus expression is reduced when SpoOJ function is impaired, and based on the *gus* gene (also called *uidA*), encoding, β-glucuronidase. In the absence of inhibitors of SpoOJ, sporulating cells of this strain produce considerably more β-glucuronidase than β-galactosidase. Inhibitors of SpoOJ would result in a dramatic change in the ratio, with decreased β-glucuronidase and increased β-galactosidase. Non-specific inhibitors affecting cell viability, ability to sporulate, activation of σ^F, or one or other of the reporter enzymes, would not produce this characteristic change.

The assay could be readily adapted to run on a high throughput basis, so as to enable the screening of large libraries of compounds. Strain 1238 would be grown in large batch culture in a hydrolysed casein growth medium and induced to sporulate by harvesting and resuspension in a starvation medium, according to standard practice (11,12). Samples of the sporulating culture would then be dispensed into the individual wells of microtitre plates containing potential inhibitors. After an appropriate period of incubation, to allow activation of σ^F and expression of the two reporter genes, the cells would be lysed and assayed simultaneously for the two enzyme products. In the case of β-galactosidase and β-glucuronidase, there are a range of substrates available for assaying the specific enzyme activities. These can give fluorescent, chemiluminescent or coloured products, which could be measured either on a continuous or a fixed time basis, using automated plate readers. Potential inhibitors could be reinvestigated in more detail using other assay methods or bacterial strains with different combinations of reporter genes. They should also produce characteristic changes in the microscopic appearance of sporulating cells (4).

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